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In re Patent Application of

LUCAS et al. Atty. Ref.: 2551-55

Appl. No. 09/779,703 Group: 1646

Filed: February 9, 2001 Examiner: O HARA

For: TNF-DERIVED PEPTIDES FOR USE IN TREATING OEDEMA

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Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

**RULE 131 DECLARATION**

I, Lucie Fransen, do hereby declare and state that:

1. I am a co-inventor of at least one claim of the above-identified application.
2. I hold a Ph.D. degree in Molecular Biology from the University of Gent, Belgium.
3. I am currently a therapeutic program manager of the Assignee of the above-identified application (i.e., INNOGENETICS N.V.) for therapeutic programs relating to immunological diseases.
4. A copy of my professional resume is attached.
5. I have reviewed the pending claims of the above-identified application.

6. I have been advised that the U.S. Patent Office official in charge of the above-identified application has indicated that the following information, if presented in the present form, would be convincing that one of ordinary skill in the art can make and use the claimed invention. I have been advised that the following information is the same as was submitted as an Annex which was filed with the U.S. Patent Office on December 2, 2003.

7. The following Examples were performed by me or under my direction and/or the analysis was performed by me or under my direction.

Example 1:

Ex vivo rat flooded lung: an artificial model of hydrostatic edema simulating the symptoms seen in heart failure.

**Description of the model**

In this model edema is induced by instillating 2 ml of 0.9% NaCl solution intratracheally. Alternatively, edema formation can be modulated by varying the height of the venous outflow and the perfusion buffer containing reservoir, or alternatively by varying the time the lung is exposed to the elevated pressure. The isolated lungs can be treated before or after edema generation by intratracheal instillation with the TNF-tip peptide (human or mouse), recombinant TNF (human, rat or mouse), Terbutaline or PBS. The clearance of alveolar fluid from the lung is calculated by means of measuring the concentration of HSA, which is added to the instillate over time. Alternatively, the over time weight loss as a measure of edema resorption (high weight loss is high liquid clearance), of the artificially instilled lung can be measured. The lung tidal volume, compliance and weight are monitored continuously during the experiment. At the end of the experiment bronchoalveolar fluid and supernatant of lung homogenate is taken for measurement of inflammatory mediators.

**Procedure**

The lungs of female Wistar rats (weight 200 – 250 g; Harlan-Winkelmann, Böchen, Germany) are prepared after terminal i.p. anesthesia by 160 mg/kg pentobarbital -Na (Merial Ltd, Halbermoos, Germany) and perfused as described by Uhlig and Wolin (1994), and by Uhlig and von Bethmann (1997). All equipment is obtained from Hugo Sachs Electronics (March-Hugstetten, Germany). Lungs are perfused at constant hydrostatic pressure (12 cm H<sub>2</sub>O) through the pulmonary artery, which resulted in a flow rate of approximately 35 ml/min. As perfusion medium, a Krebs-Henseleit buffer (38°C) containing 2% bovine serum albumin (Fraction V, Serva, Heidelberg, Germany), 0.1% glucose (Riedel-de-Haen Inc., Seelze, Germany), and 0.3% HEPES (ICN Biomedicals Inc., Ohio, USA) is used. The total amount of recirculating buffer is 100 ml. The lungs are suspended by the trachea and ventilated by negative pressure ventilation (inspiratory pressure: -7 cm H<sub>2</sub>O,

expiratory pressure: -2 cm H<sub>2</sub>O) with 80 breaths per minute resulting in a tidal volume of approximately 2 ml. Every 5 minutes, a deep inspiratory breath (-20 cm H<sub>2</sub>O) is performed. Artificial thorax chamber pressure is measured with a differential pressure transducer (Validyne DP 45-14), and an air flow velocity with a pneumotachograph tube (Fleisch Type 0000) connected to a differential pressure transducer (Validyne DP 45-15). The perfusate flow (Narcomatic RT-500) and the arterial and venous pressure (Statham P23BB) are continuously monitored. The pH of the perfusate before entering the lung is kept at 7.25 to 7.35 by automatic bubbling of the buffer with CO<sub>2</sub> as soon as the pH exceeds this range. A weight transducer is integrated into the chamber lid which allows the continuous assessment of lung weight. Data are recorded on a Pentium II computer using the Matlab Software package (Mathworks, Inc., Natick MA, USA). For lung mechanics, the data are analyzed by applying the following formula:

$$P = 1/C * V_T + R_L dV/dT$$

Where P is chamber pressure, C pulmonary compliance, V<sub>T</sub> tidal volume, and R<sub>L</sub> airway resistance. All lung physiology parameters were normalized to time point 0, i.e. after the end of the preconditioning perfusion of 40 minutes.

## Results

Tables 1 and 2 summarize the weight loss of the ex vivo treated lung measured either 100 minutes or 80 minutes after instillation.

The following treatments were applied for the weight measurement at 100 minutes after instillation:

- control treatment with saline
- 1 mM Terbutalin either alone or in combination with 10 µM Amiloride or 500 µg Chitobiose
- recombinant human TNF at a concentration of 5 µg/lung either alone or in combination with 10 µM amiloride, 500 µg Chitobiose or 500 µg cellobiose
- Synthetic human TNF-tip peptides at a concentration of 1000 µg/lung (either cyclic hum Ltip: CGQRETPEGAEAKPWYC, the short peptide: CTPEGAEAC (STip) or the cyclic mutant peptide: CGQREAPAGAAAKPWYC (hum mut Tip)).

For the weight loss assessment after 80 minutes, the following treatments were compared:

- control treatment with saline
- 1 mM Terbutalin either alone or in combination with 10  $\mu$ M Amiloride
- recombinant human TNF at a concentration of 5  $\mu$ g/lung either alone or in combination with 10  $\mu$ M Amiloride or 500  $\mu$ g chitobiose
- recombinant mouse TNF or rat TNF at a concentration of 5  $\mu$ g/lung
- Synthetic human TNF-tip peptides at a concentration of 1000  $\mu$ g/lung (either cyclic hum Ltip, the linear hum Ltip: CGQRETPEGAEAKPWY, the STip, or the cyclic mutant peptide (hum mut Tip)).

The results clearly demonstrate that terbutaline, TNF as well as the synthetic peptide is capable of clearing alveolar fluid content ex vivo after intratracheal instillation of the molecule. The effect can be specifically blocked by co-treatment with amiloride or chitobiose but not by cellobiose. The lectin-deficient peptide (hum mut Tip) wherein the TPEGAE is replaced by APAGAA is inactive in clearing alveolar fluid content.

The data also demonstrate that the cyclic long, the linear long and the short peptide are equally active in fluid clearance when measured 80 minutes after instillation. The linear peptide is less active when measuring for 100 minutes after instillation.

Example 2:

Rat model of lung reperfusion injury (warm ischemia and reperfusion): a model of Acute Respiratory Distress Syndrome (ARDS).

**Description of the model**

In a modification of the model of Ohno *et al.* (1993) male Fischer (F344) rats (200 – 250 g) undergo clamping of the left pulmonary artery, pulmonary vein and main bronchus for 36 minutes of warm ischemia. After reperfusion of the left lung, the right lung is occluded to assess the function of the left lung for 90 minutes. The peptide is given at 3 minutes after reperfusion by either instillation or intravenous injection via the subclavian vein.

**Table 1**  
Weight loss (mg) of ex vivo instilled rat lung over 100 min.

	control NaCl	Tertbutaline 1 mM	IL TNF S. 50 ng/lung	cyclic human L tip	S Tip	human mut Tip
Anilofrone 10 $\mu$ M	X	X	X	X	X	X
Clinidbiase 500 $\mu$ g	488	1083	197	895	347	580
Cellulose 500 $\mu$ g	420	805	466	968	288	384
	571	1223	89	990	653	41
	612	991			639	
	365	1425			786	
	461	1370			968	
	402	1023			758	
	534	1646			703	
	516				804	
	539				639	
	260					
	590					

Table 2  
Weight loss (mg) of ex vivo instilled rat lung over 80 min

Amiloride 10 $\mu$ M Chlathiose 500 $\mu$ g	control NaCl		Terbutalin 1 mM X		ratTNF $\zeta$ juggling X		ratTNF 5 $\mu$ g/lung N		ratTNF ratTNF 5 $\mu$ g/lung X		ratTNF cyclic human lung tip X		ratTNF linear lung tip X		ratTNF human lung tip X		ratTNF human lung tip X	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
411	830	61	344	351	530	722	516	900	462	703	625	625	411	411	411	411	411	411
379	702	439	672	251	297	584	809	918	498	744	231	231	379	379	379	379	379	379
489	1035	60	580	23	448	589	658	498	507	507	234	234	489	489	489	489	489	489
507	872	156	1105					585	863	607	500	500	507	507	507	507	507	507
388	1219	427	584					661	585	781	232	232	388	388	388	388	388	388
425	1119		579					648		541	541	541	425	425	425	425	425	425
393	822		680					575					393	393	393	393	393	393
493	1075		877					601					493	493	493	493	493	493
480	675		649					585					480	480	480	480	480	480
388	730		955					648					388	388	388	388	388	388
251	1000		607					575					251	251	251	251	251	251
200	923		607					931					200	200	200	200	200	200
450													450	450	450	450	450	450
220													220	220	220	220	220	220
530													530	530	530	530	530	530
470																		

**Procedure**

1. Anaesthesia of the rat in a glass chamber, 4% Halothane and Oxygen 4/min.
2. Intubation of the rat with a 1.8 mm catheter.
3. Shaving the fur on the abdomen and left side of the animal.
4. Ventilation via the tracheal tube 100/min, 2.5 ml 20% oxygen, Harvard Rodent Ventilator (Harvard Apparatus, South Natick, Massachusetts), PEEP 5 mm H<sub>2</sub>O.
5. A left thoracotomy is carried out in the in 4<sup>th</sup> intercostal space.
6. Dissection of the left lung hilar region.
7. Microvascular clips are placed around the left pulmonary artery (PA), Pulmonary vein (PV) and left main bronchus (B) to induce warm ischemia of the left lung. Small circulation is still ongoing through the right lung. This is the start of the Warm Ischemia Time (WIT).
8. Anaesthesia is maintained with 2% Halothane, 40% Oxygen.
9. A provisory Z suture is made to temporary close the thoracotomy and a gaze sponge is put on the animal to decrease warmth loss.
10. Near the end of the WIT, a thoracophrenolaparatomy in the midline with thorough electrocaterisation. (two peans on the sternum).
11. Putting 2-0 Softsilk on the trachea around the intubation tube.
12. Dissection of the right hilar elements.
13. Taking away microvascular clips from the left hilar elements. End of WIT of the left lung after 36 minutes.
14. Putting microvascular clips on right PA and B. Small circulation only through left lung. Start developing of left oedema time (LLOT).
15. After 3 minutes of LLOT: instillation of the drug in 0.5 ml of 0.9% saline solution (Baxter) or injection of the drug in 0.25-0.3 ml of 0.9% saline through the subclavian vein.
16. After 6, 30, 60 and 90 minutes of LLOT; puncture of the Aortic Arch (AA) with needle for blood gas analysis in Radiometer ABL 700 Serie, (Denmark).
17. After each puncture the AA puncture hole is pressed with a cotton swab.
18. Blood gas analysis is taken every time from AA (the heart rate should be over 60).
19. After the last blood gas analysis (90 minutes), both lungs are flushed via the PA with 20 ml of 0.9% saline at a pressure of 20 cm H<sub>2</sub>O. Thereto the right atrium and vena cava inferior are incised and a rubber hose is inserted into the PA main steam. The heart-lung block is excised for storage under formaline or in the freezer awaiting

immunohistopathological analysis. Alternatively, the lung can be excised without flushing to assess wet to dry ratio's.

### Results (Fig. 1)

The graph represents the pO<sub>2</sub> levels that can be measured over time at the start of the LLOT in the differently treated animals. The control line (either no treatment or saline treatment) clearly shows the severity of the treatment since only very low pO<sub>2</sub> levels can be measured (below 100 mmHg) and the animals have clearly breathing problems since their lungs are filled with water. Animals treated with the control peptide (hum mut Tip: CGQREAPAGAAAKPWYC with a disulfide bond between Cys at position 1 and position 17 and wherein the TPEGAE of the sequence of the peptide was replaced by APAGAA and wherein the lectin binding activity and membrane conductance activity (patch clamp) was lost) followed completely the values seen in the control animals.

The cyclic hum Ltip peptide: CGQRETPEGAEAKPWYC (with a disulfide bond between Cys at position 1 and position 17) treated animals (and this over a broad dosage range, extending from 500 µg/kg to 5 µg/kg, have significantly much better pO<sub>2</sub> levels and lower pCO<sub>2</sub> levels (data not shown) and are less suffering from fluid overload.

Interestingly, this is also a very rapid effect since better pO<sub>2</sub> levels can already be measured at the first time point at six minutes after reperfusion.

In the warm ischaemia reperfusion model a significantly increased gain in pO<sub>2</sub> blood gas could be observed upon iv application of the synthetic peptide while no effect could be seen when treated with the synthetic lectin deficient control peptide or in untreated animals.

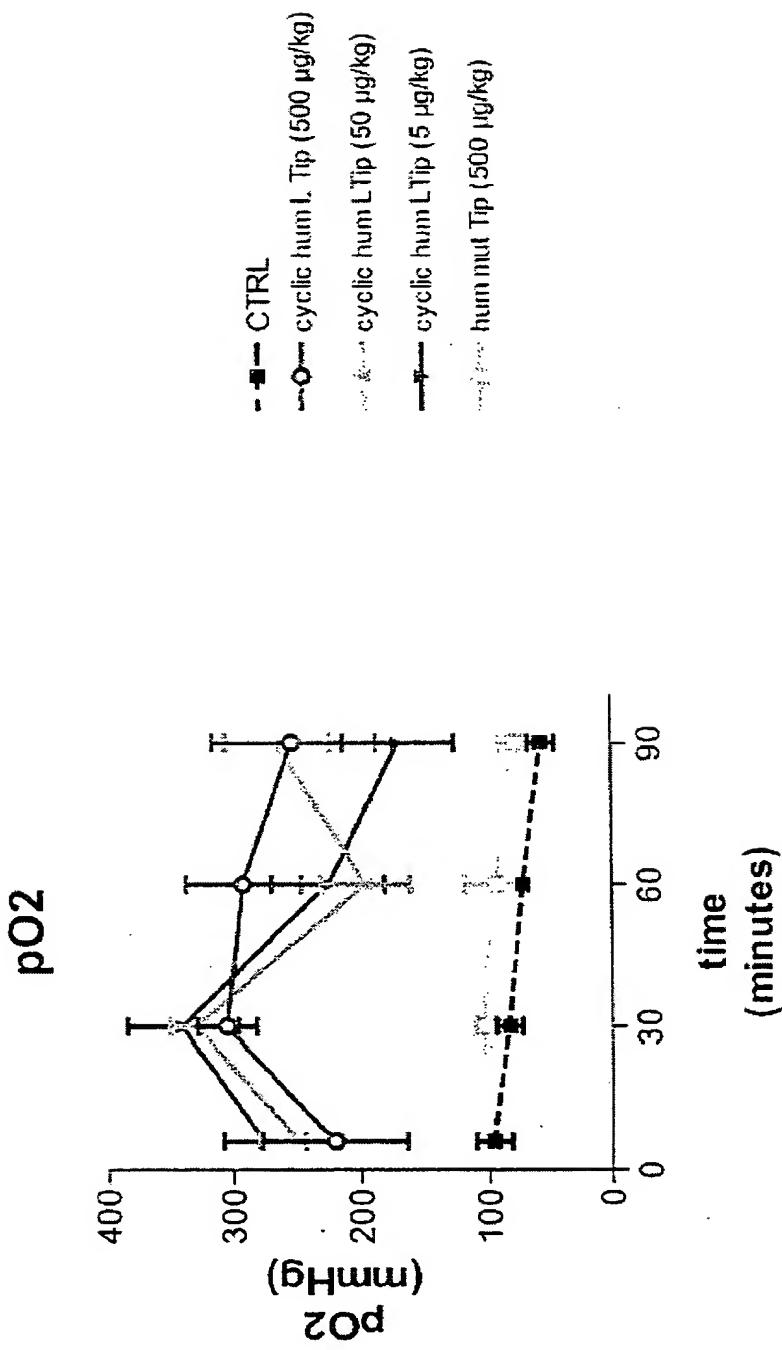


Figure 1:

Effect on  $pO_2$  blood gases (in mmHg) of different dosages of cyclic hum L tip ( $\circ$ ; 500  $\mu\text{g}/\text{kg}$ ;  $\blacktriangle$ ; 50  $\mu\text{g}/\text{kg}$ ;  $\blacktriangledown$ ; 5  $\mu\text{g}/\text{kg}$ ) and ctrl values (saline treatment  $\blacksquare$ ) or cyclic hum mut tip ( $\blacksquare$ ; 500  $\mu\text{g}/\text{kg}$ ) at time point 6, 30, 60 and 90 minutes after the start of LLOT.

Example 3:

Single left lung transplantation in syngeneic rats : a model of lung transplantation

**Description of the model**

Female Wistar or Fischer rats or weighting 250 to 260 g undergo orthotopic single left lung transplantation after 20 hours cold ischemia using a cuff technique for the vessel anastomoses and a conventional running suture for the bronchial anastomosis.

**Procedure**

Donor procedure: Animals are anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg) and heparinized (500 I.U./kg). A tracheotomy is performed and the animals are ventilated through a cannula with 100% O<sub>2</sub> by a Harvard rodent ventilator (Harvard apparatus, South Natick, Massachusetts) at a tidal volume of 10 ml/kg. After cutting the inferior vena cava and resection of the left appendix of the heart, a small silicon hose is inserted into the main pulmonary artery. Both lungs are flushed with 20 cc of low-potassium dextran-solution (LPD) (Perfadex®, Kabi Pharmacia, Sweden) at a pressure of 20 cm H<sub>2</sub>O. The trachea is then tied in end-inspiration. After removal of the heart-lung block, 14 gauge cuffs are placed around the pulmonary artery and vein, and the vessels are inverted and tied onto the cuff. The lung is stored in LPD solution at 4°C until implantation.

Recipient procedure: Transplantation was performed after 20 hours of cold ischemia (4°C). The recipient is anesthetized by breathing Halothane in a glass chamber, intubated, and anesthesia is maintained with Halothane 2%. A left lateral thoracotomy is performed in the 4<sup>th</sup> intercostal space. The left hilum is dissected. After clamping the pulmonary artery and vein with removable microclips, the pulmonary vein is opened, flushed with heparinized saline solution, and the cuff is inserted and fixed with 6-0 Silk. In the same way, the pulmonary artery is anastomosed. The native left lung is removed and the bronchial anastomosis performed with a running over-and over suture with 9-0 Monosof® (Autosuture, Switzerland). The lung is first re-ventilated and then reperfused. A chest tube is inserted and the thoracotomy closed. The chest tube is removed after restoration of sufficient spontaneous breathing.

Typically, the animals tolerate the procedure well and start eating about one hour after extubation.

Administration of the drug: The synthetic peptide was administered by instillation in the left main bronchus of the donor lung via a little cut in the most proximal part of the bronchus, 20 minutes before transplantation in a total volume of 0.5 ml of 0.9% saline. The peptide could also be applied intravenously to the recipient animal directly after transplantation. Both treatments (instillation of the donor lung and intravenous application to the recipient animal) could also be combined.

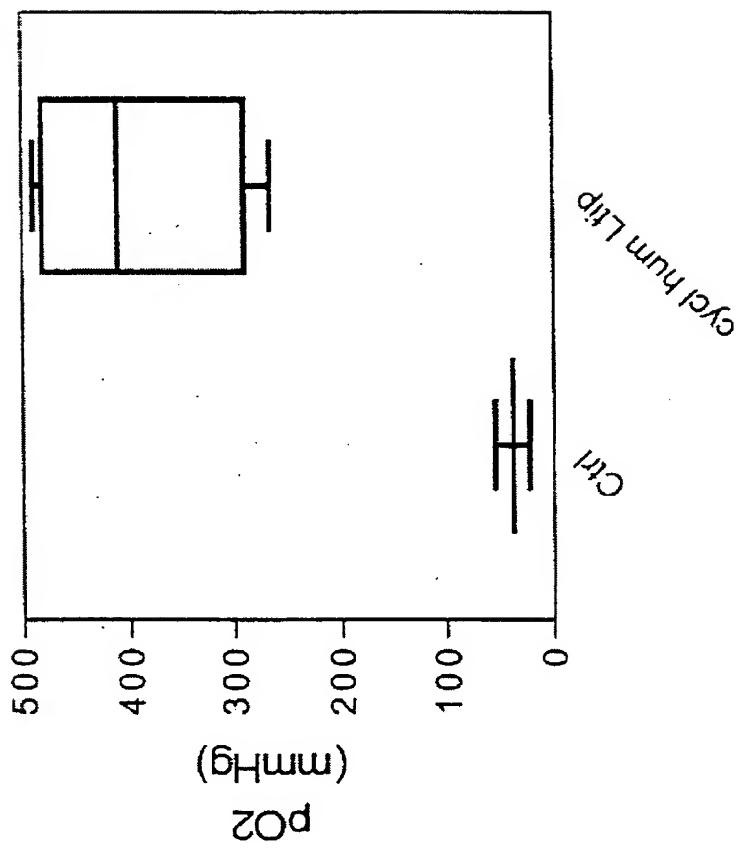
Alternatively, the peptide can also be applied in the perfusate during the perfusion of the donor lung.

Assessment: - Arterial blood gas analysis:

Recipient animals were anesthetized 24 hours after reperfusion. Each animal was ventilated with an FIO<sub>2</sub> of 1.0, a frequency of 100 breaths/minute, and a tidal volume of 8 ml/kg body weight by a tracheotomy. For functional assessment of the transplanted left lung, the right hilum was dissected and the right pulmonary artery and right main bronchus were occluded with microvessel clips. Five minutes after occlusion, a steady state was reached and an arterial blood gas sample was drawn from the thoracic aorta.

**Results (Fig. 2)**

In the model, pO<sub>2</sub> levels of the transplanted control animals (either no treatment or treatment with saline) are low laying around 56 +/- 7 mmHg. As can be clearly seen from the elevated blood gases, the cyclic hum LTip (with a disulfide bond between Cys at position 1 and position 17) given by intratracheal application at a concentration of 500 µg/kg restores very efficiently the lung function of the donor lung. Normal pO<sub>2</sub> values of 395 +/- 95 mmHg are reached by pre-treatment of the donor lung with the TNF-tip peptide.



**Figure 2:**  
pO<sub>2</sub> blood gases (in mmHg) of lung transplanted rats measured 24 hours after transplantation. Donor lung was treated by instillation of the drug into the bronchus 20 minutes before transplantation with ctrl (untreated transplanted animal) or 500 µg/kg of cyclic lung Lip.

Example 4:

Identification of the alveolar edema reabsorption activity of Tumor Necrosis Factor

**MATERIALS AND METHODS**

*TNF and TNF-derived peptides.*

*Escherichia coli*-derived recombinant murine TNF (further referred to as TNF in the text) was synthesized as described by Lucas *et al.*, 1994 and Lucas *et al.*, 1997. A long tip peptide 99-115 (cyclic mu Ltip) was synthesized with the use of Fmoc- $\alpha$ -amino group protection (Fields *et al.*, 1990) and purified with a C18 reversed-phase high-performance liquid chromatography column.

To retain the original TNF conformation as much as possible, Ltip peptide was circularized.

Ser<sup>99</sup> of the TNF sequence was replaced by Cys, and Cys<sup>100</sup> by Gly so that the disulfide bridge could be formed between Cys<sup>99</sup> and Cys<sup>115</sup> in the peptide. As a control peptide, a scrambled tip peptide was used, consisting of the same amino acid composition as the tip peptide, but in a random order.

Cyclic mu Ltip: CGPKDTPEGAEELKPWYC

Cyclic mu scrambl Tip: CGTKPWELGPDEKPAYC

*Animals and animal preparation.*

Mice. Male C57BL/6 TNF-R1 /TNF-R2 double knock-out mice (Bruce *et al.*, 1996) ( $n = 8$ , 20-30 g) and C57/BL6 wild type mice ( $n = 8$ , 20-30g) were purchased from the Jackson laboratory (Bar Harbor, ME, USA). These animals were housed in air-filtered, temperature-controlled units with food and water. All procedures were approved by the University of California San Francisco Committee on Animal Research.

In situ mouse model. Mice were killed by an overdose of pentobarbital sodium (200 mg/kg i.p.). A tracheotomy was done with a 20-gauge trimmed angiocath plastic needle (Becton Dickinson, Sandy, UT, USA). The lungs were inflated with 7 cm H<sub>2</sub>O continuous positive airway pressure (CPAP) with 100 % oxygen throughout the experiments. Body temperature was maintained at 37-38°C by an infra-red lamp placed 30 cm above the body (Fisher, Santa Clara, CA, USA). The lamp was cycled on and off to maintain the core temperature. A

temperature probe (Yellow Springs Instrument, Yellow Springs, OH, USA) was inserted via a 0.5 cm incision into the abdominal cavity to monitor the core temperature throughout the experiment. These methods have been reported in Matthay *et al.*, 1996 and Ma *et al.*, 2000. The instillate consisted of 5 % bovine serum albumin (Sigma Chemical, St. Louis, MO) with Ringer's lactate that was adjusted to be isoosmolar with mouse plasma (Matthay *et al.*, 1996 and Ma *et al.*, 2000). Wild type mTNF (0.5 µg/mice) was instilled into the distal lung. We added 0.1 µCi of <sup>131</sup>I-labelled albumin (Merck-Frost, Montreal, PQ, Canada) to the instillate as a labeled alveolar protein tracer.

*Group 1:* Basal alveolar fluid clearance in the wild type mice (n = 3) and TNF-R1/TNF-R2 double knock out mice (n = 3).

*Group 2:* Effect of wild type mTNF (0.5 µg/mouse) stimulated alveolar fluid clearance was measured in wild type mice (n = 5) and TNF-R1/TNF-R2 double knock out mice (n = 5) (Bruce *et al.*, 1996). According to our previous studies, alveolar fluid clearance over 15 min was measured by the increase in the final concentration of the alveolar protein tracer compared with the initial instilled tracer protein concentrations (Matthay *et al.*, 1996; Ma *et al.*, 2000).

Rats. For the *ex vivo* experiments, 31 Sprague-Dawley rats (300 to 350 g) were tracheotomized and mechanically ventilated (40% oxygen in air) under isoflurane anesthesia. The experimental protocol was reviewed and approved by the Ethics Committee for Animal Research and by the Veterinary Office of our institution. The femoral vessels were cannulated for blood sampling, fluid replacement, and continuous arterial blood pressure monitoring. Following anticoagulation (1.5 IU/g i.v. heparin), 20 ml of blood were withdrawn and replaced by an equal volume of dextran-40 (Macrodex 10% in normosaline) to serve as priming volume for the isolated perfusion circuit. The heart-lung block was prepared as described by Ma *et al.*, 2000, weighed and assigned to one of four groups. Each received a pretreatment consisting of either 0.9% NaCl (n = 12), TNFα (5 µg; n = 8), cyclic mu Ltip (1 mg; n = 7) or cyclic mu scrambl Tip (1 mg; n = 4), injected as 500 µl aliquots into the trachea at about 1 cm above the carena, 5-10 minutes before starting reperfusion. The lungs were suspended, mechanically ventilated, and perfused at constant pressure with autologous blood as described by Ma *et al.*, 2000.

An inspiratory and expiratory quasi-static pressure-volume (PV) curve was performed at the end of the 2-hour reperfusion period by inflating and then deflating the lungs at a constant rate (0.3 ml/sec) using an automated infusion pump. A sigh was applied in all groups every 15

minutes to minimize atelectasis formation. The blood pH was maintained between 7.3 and 7.5 and, if necessary, corrected with sodium bicarbonate 8.4% or a change of the inspired CO<sub>2</sub> as required by the blood gas analysis. Every 30 minutes, a blood sample was collected for blood gas analysis as well as for hematocrit and electrolyte concentration.

Inclusion criteria. Immediately following the start of reperfusion, the preparation had to fulfill three technical inclusion criteria:

- 1) left atrial pressure (LAP) 5 - 10 mmHg;
- 2) peak airway pressure (AWP) < 15 cmH<sub>2</sub>O; and
- 3) pulmonary blood flow (PBF) > 10 ml/min.

Subsequently, the included lungs that did not remain isogravimetric within 30 min of perfusion were excluded. The included lungs were then flooded with gentle intratracheal instillation of 2 ml normal saline and the recorded variables measured for 2 subsequent hours.

Histological analysis. Random lung samples were fixed for light and electron microscopy and analyzed by pathologists blinded to the lung's protocol group assignment.

Statistical analyses. A two-way ANOVA was used to compare data between and within groups (repeated measures design), followed by Duncan's multiple comparisons test if the analysis of variance resulted in a p-value < 0.05.

## RESULTS

### TNF mediates fluid resorption in the *in situ* mouse lung model by means of a TNF receptor-independent mechanism.

Wild type TNF in C57/BL6 mice increased alveolar fluid clearance (AFC) by 24% ( $p<0.05$ ) over 15 min in the *in situ* mice compared to controls (Figure 3). TNF-R1/R2<sup>-/-</sup> C57/BL6 mice showed an equivalent increase in AFC in control conditions as well as in the presence of TNF, strongly indicating that receptor-independent effects of TNF are responsible for the enhanced fluid clearance in this model in mice.

**Cyclic mu Ltip induces a significant weight loss in flooded perfused rat lungs.**

In order to validate the hypothesis that receptor-independent effects predominate in the fluid resorption capacity of TNF, we compared the effects of an intratracheal pretreatment with (1) cyclic mu Ltip, a 17 amino acid mouse TNF-derived peptide that does not bind to the TNF- $\alpha$  receptors and that mimics the  $\text{Na}^+$ -channel activating effect of TNF or (2) a scrambled peptide (cyclic mu scrambl Tip) displaying the same amino acids but in a random order, with (3) the native protein in a volume-controlled isolated blood perfused rat lung model. Based on relative potencies in activating sodium currents *in vitro* (Hribar *et al.*, 1999), the lungs were treated with 5  $\mu\text{g}$  of TNF and with 1  $\text{mg}$  of the peptides.

During the first 15 minutes of reperfusion, lung weight increased similarly in the four treatment groups, due to the filling of the pulmonary vasculature imposed by the respective predetermined vascular (MPAP and LAP) and airway pressure gradients (AWP and PEEP) (NaCl-group:  $+0.69 \pm 0.46$  g; TNF-group  $+0.87 \pm 0.60$  g; cyclic mu Ltip-group:  $+0.80 \pm 0.61$  g; cyclic mu scrambl Tip-group:  $+0.71 \pm 0.50$  g). Following stable perfusion conditions for 15 minutes, alveolar flooding by intratracheal instillation of 2 ml normal saline (time 0 in Figure 4) produced a further acute increase in lung weight of 1.9 g without modifying the perfusate's characteristics and pulmonary hemodynamics. In contrast, there was a marked change in dynamic lung mechanics observed similarly in all treatment groups, demonstrated by a two-fold increase in peak insufflation pressure, a 60% reduction in dynamic lung compliance ( $C_{dyn}$ ), and a 75% increase in expiratory airway resistance ( $R_{aw}$ ) (Figure 4).

Following alveolar flooding, lung weight increased slightly though not significantly over time in NaCl-treated lungs (mean weight gain,  $+0.28 \pm 0.09$  g). In contrast, lungs pretreated with the cyclic mu Ltip peptide progressively decreased in weight ( $p < 0.001$ ), and were statistically different from saline-treated lungs after 45 minutes of reperfusion (Figure 4). At 2 hours, the lungs in this group had lost approximately half of the intratracheal instilled saline. In contrast, lungs pretreated with TNF or cyclic mu scrambl Tip peptide showed no significant change in weight over time.

#### **Improvement of respiratory parameters upon cyclic mu Ltip treatment.**

Peak AWP,  $C_{dyn}$ , and  $R_{aw}$  of the NaCl-pretreated group remained stable after alveolar flooding, showing no significant change over time (Figure 4). Similarly, for the TNF- $\alpha$ - and cyclic mu scrambl Tip-pretreated lungs there was no difference from the saline-treated group. In contrast, cyclic mu Ltip-pretreated lungs showed a progressive improvement in lung mechanics following alveolar flooding during reperfusion ( $p < 0.001$  for all three measured

variables) (Figure 4). These changes were statistically significant compared to NaCl-treated lungs after 15 minutes for  $C_{dyn}$ , and at 60 minutes for peak AWP, but did not reach statistical significance for  $R_{aw}$ .

Figure 5 shows the quasi-static airway PV curves obtained at the end of the study. In NaCl-treated lungs, alveolar flooding produced a severe decrease in the slope of the initial inspiratory limb of the curve (i.e. a reduced static inspiratory lung compliance), up to a sharp inflection point situated at  $22.6 \pm 1.9$  cm H<sub>2</sub>O obtained after only 1.5 ml of inflated volume. Thereafter, a continuing rise in inflation pressure with volume opened the flooded and collapsed lung abruptly. The expiratory limb of the PV curve was not altered compared to control normal lungs (data not shown). Pretreatment of the lungs with TNF- $\alpha$  did not significantly influence the initial slope of the PV curve, but shifted the second part to the left (lower inflection point,  $20.0 \pm 0.8$  cmH<sub>2</sub>O;  $p < 0.05$  compared to the NaCl group). Pretreatment with cyclic mu Ltip consistently ameliorated the whole inspiratory curve, with a lower inflection point at  $17.2 \pm 1.6$  cmH<sub>2</sub>O ( $p < 0.05$  compared to both the NaCl and TNF groups) obtained at an inflation volume of  $3.4 \pm 0.9$  ml denoting an improved static inspiratory lung compliance. The expiratory part of the curve was not influenced by TNF- $\alpha$  or cyclic mu Ltip pretreatment, indicating that once fully recruited, the lung recovers its normal elastic recoil properties manifested during the deflation curve, that is independent of the amount of alveolar fluid.

**Cyclic mu Ltip does not cause leukocyte sequestration in the treated lungs.**

As shown in Figure 6b, hematoxylin-stained slices of lungs pretreated with mTNF- $\alpha$  showed a significantly increased leukocyte infiltration, as compared to controls (Figure 6a). In contrast, lungs pretreated with cyclic mu Ltip did not show this increased leukocyte infiltration (Figure 6c), indicating that, in contrast to TNF- $\alpha$ , cyclic mu Ltip peptide does not exert a pro-inflammatory reaction in the lung.

Electron microscopy analysis of TNF- $\alpha$ -pretreated lungs reveals prominent numerous endothelial flaps (white arrow) protruding in the vascular lumen around an erythrocyte (E), numerous cytoplasmic blebs of the pneumocyte I (black arrow BA), and tubular myelin surfactant (S) in the alveolar space (Figure 7a). This particular microscopic pattern is not found in cyclic mu Ltip-treated lungs (Figure 7b) and is also less evident in cyclic mu scrambl Tip-treated or saline treated lungs (Figure 7c,d) compared to TNF- $\alpha$  instillation (Figure 7a).

In all analyzed lungs, there is an increased number of pinocytic vesicles in endothelial cells and pneumocytes.

## DISCUSSION

Active  $\text{Na}^+$  transport across the alveolar epithelium *in vivo* was proposed to help the reabsorption of fetal fluid after birth and to keep the adult alveolar spaces free of fluid, especially when alveolar permeability to plasma proteins has been increased (Matthay *et al.*, 1996). Epithelial  $\text{Na}^+$  channels represent the rate-limiting step in  $\text{Na}^+$  absorption (Matalon *et al.*, 1999; Hummler *et al.*, 1999). Different types of channels have been described on alveolar type II epithelial cells and fetal distal lung epithelial (FDLE) cells. The results of *in vivo* and *in vitro* studies indicate that  $\text{Na}^+$  ions in the alveolar lining fluid passively diffuse into FDLE and alveolar type II cells through non-selective cationic channels and  $\text{Na}^+$  selective, amiloride-sensitive channels located in their apical membrane. The favorable electrochemical driving force for  $\text{Na}^+$  influx is maintained by the ouabain-sensitive basolateral  $\text{Na}^+-\text{K}^+$ -ATPase that also transports  $\text{Na}^+$  into the interstitial space (Matalon *et al.*, 1999).

Hydrostatic pulmonary edema is a common complication of congestive heart failure, resulting in substantial morbidity and mortality (Koerner *et al.*, 2001; Fromm *et al.*, 1995). In addition, acute pulmonary edema or pulmonary reimplantation response frequently occurs after lung transplantation (Khan *et al.*, 1999), and is caused by ischemic vascular injury of the allograft, resulting in increased permeability of the lung after reperfusion, in turn leading to interstitial and alveolar edema. Most patients with ARDS or acute lung injury also have a dramatically decreased edema resorption capacity, correlating with morbidity and higher mortality (Ware *et al.*, 2001).

Recently,  $\beta 2$ -adrenergic agonists, such as terbutaline, have been shown to resolve hydrostatic edema very efficiently in both sheep and rat models (Frank *et al.*, 2000). However, long-term  $\beta 2$  adrenoceptor agonist therapy leads to a desensitization of  $\beta 2$  adrenoceptor-mediated cardiovascular and noncardiovascular effects in humans *in vivo* (Poller *et al.*, 1998) and may lead to tachyphylaxis in asthmatic patients (Brodde *et al.*, 1985). Therefore, in these patients there should be an evaluation of alternative agents. The TNF-derived tip peptide could represent such an alternative, since it is not likely to interfere with  $\beta 2$ -adrenoreceptors and it may activate sodium channels in type II lung epithelial cells. At this time, the potential effect on type I alveolar epithelial cells can however not be excluded.

TNF was shown to increase sodium uptake in the A549 type II alveolar epithelial cell line (Fukuda *et al.*, 2001). This effect was suggested to imply both TNF $\alpha$  receptor-dependent and -independent activities. Indeed, on the one hand antibodies directed against TNF-R1 and TNF-R2 efficiently blocked this effect, but on the other hand, a mouse TNF mutant lacking its lectin-like activity, which still efficiently mediates most of the receptor-mediated effects (Lucas *et al.*, 1997), lacked the sodium channel activating effect *in vitro* and when given to rats (Fukuda *et al.*, 2001). The results in this study in an *in vivo* mouse model and an *ex vivo* rat lung model indicate that TNF receptor-independent effects predominate in the cytokine's fluid resorption activity. Indeed, in mice that lacked both TNF receptors (Bruce *et al.*, 1996), mouse TNF had the same efficiency in increasing fluid resorption as in wild type animals. Moreover, the Ltip peptide efficiently induced weight loss in the *ex vivo* flooded perfused rat lung, without exerting the TNF receptor-mediated pro-inflammatory activities that lead to leukocyte sequestration.

Physiologic, clinically relevant parameters were measured as indirect evaluation of edema clearance in the model of isolated, ventilated and blood perfused rat lung. After alveolar flooding, peak inspiratory pressure immediately increased in the lungs in all groups and provided an indirect indication of the volume of edema remaining in the alveoli. Increased intratracheal pressures during mechanical, constant volume ventilation can reflect bronchoconstriction, atelectasis formation, pulmonary edema, or restricted lung volume that appears after alveolar flooding.

The isolated perfused lung is subject to atelectasis and we therefore chose to apply a sigh every 15 minutes. We have no reason to suspect a bronchoconstrictive phenomenon in this model which lacked physiological innervation, and the amelioration of intra-tracheal pressure in the treated group suggests an effect of the cyclic mu Ltip peptide on the amount of alveolar edema. The dynamic compliance and airway resistance are other indirect but clinically relevant measurements which demonstrated the efficacy of the pre-treatment with the TNF-tip peptide compared to both TNF and control pre-treatments.

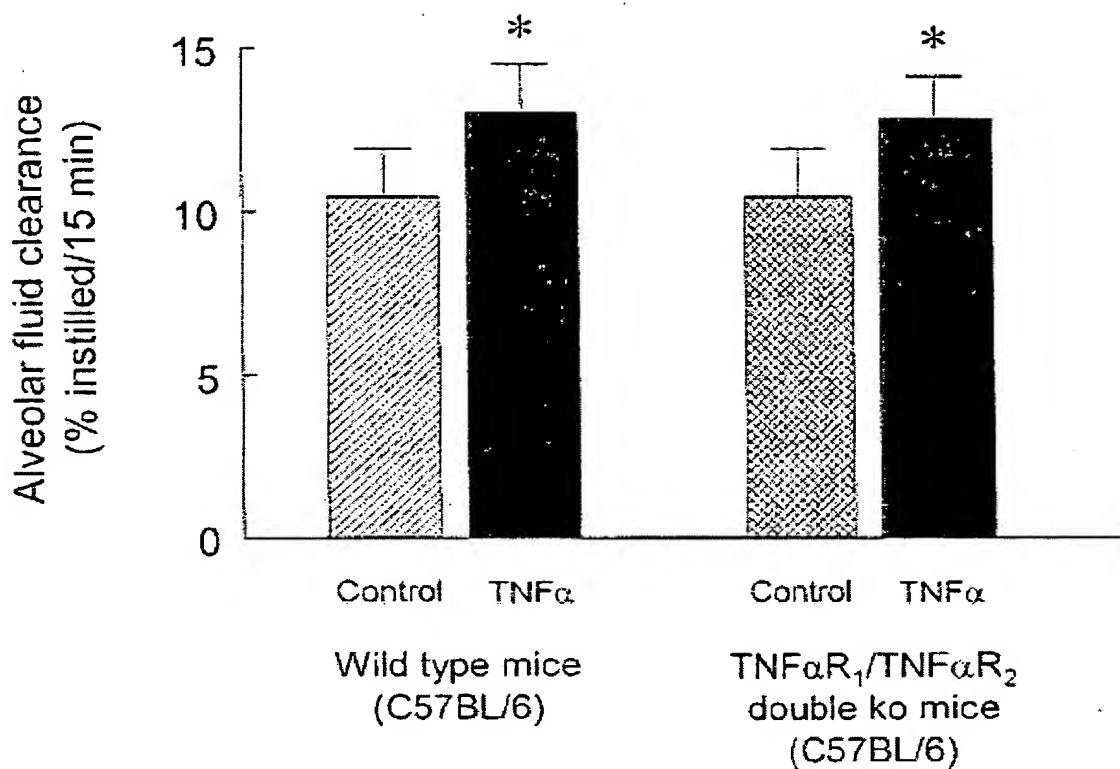
The change in lung weight is another method for measuring lung edema clearance. The advantage of using an isolated lung was the ability to continuously measure the lung weight throughout the experiment. The lung weight varies depending on the amount of vessels recruited and filled with blood. Our model allowed us to maintain this vascular recruitment constant throughout the experiment by fixing and controlling both the perfusion pressure and the left atrial pressure. Evaporation was minimized by humidifying the lung chamber.

The weight loss associated with cyclic mu Ltip pretreatment correlated with the reduced intratracheal pressures, the partial recovery of baseline lung dynamic compliance and therefore with an increase in alveolar fluid clearance. The continuous monitoring of all clinical parameters showed a continuous amelioration of the lung mechanics of the peptide-treated group throughout the entire experiment.

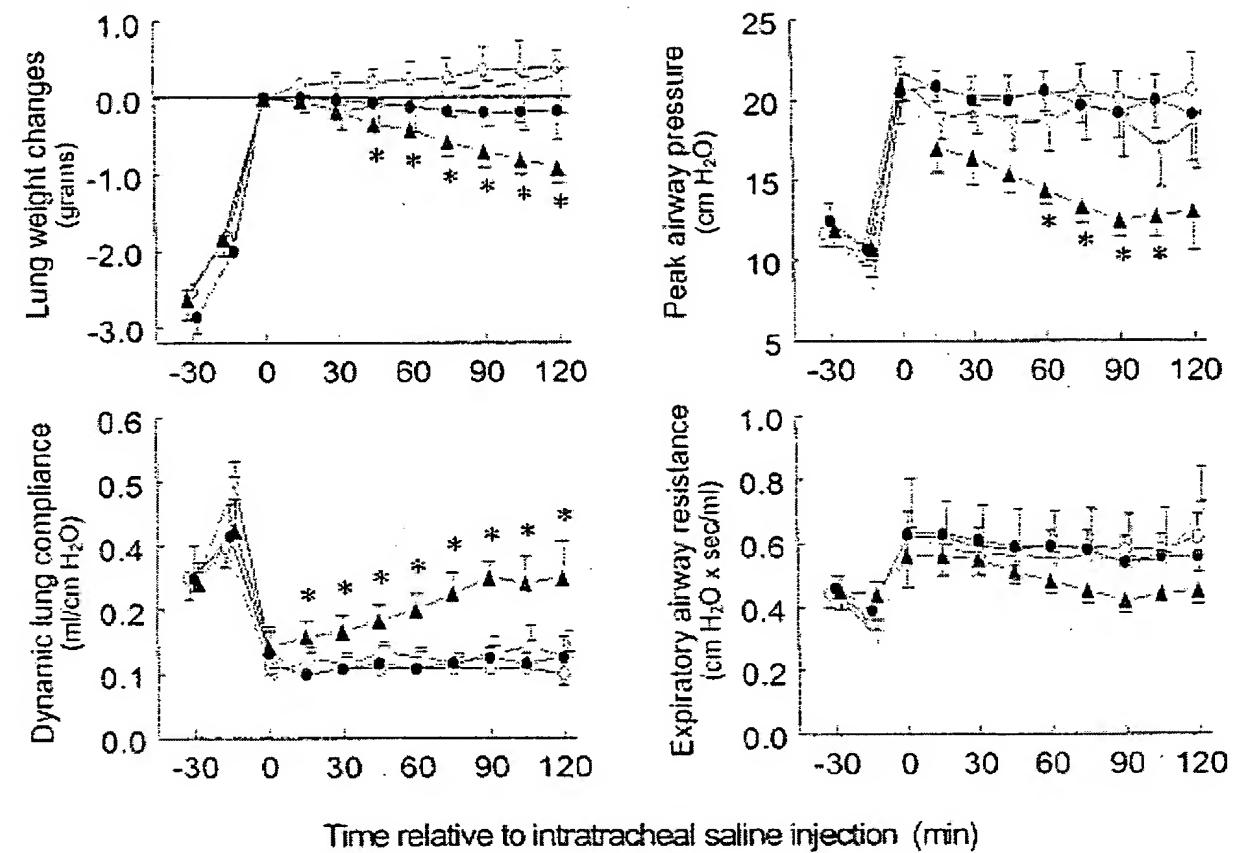
One major observation of this study was that cyclic mu Ltip peptide showed an edema resorption effect, whereas TNF was only slightly different from NaCl.

The tip peptide of TNF was shown not to exert the TNF receptor-mediated pro-inflammatory activities, such as upregulation of ICAM-1, or induction of E selectin in microvascular endothelial cells (Hribar *et al.*, 1999). Moreover, in a parallel study using a Krebs-Henseleit buffer perfused isolated flooded rat lung model, we could show a significant fluid reabsorption activity of TNF, indicating that in the isolated rat lung, blood components can inhibit or counter-act this cytokine effect (Braun *et al.*, manuscript in preparation).

In conclusion, this study indicates that receptor-independent activities of TNF, mediated by its lectin-like domain, predominate in its edema resorption activity in a model of alveolar edema in mice and rats.



**Figure 3:**  
Effect of TNF-R1/TNF-R2 double knock-out on basal and TNF-stimulated alveolar fluid clearance in mice. \*p>0.05 versus basal clearance.



**Figure 4:**  
Effect of intratracheal pretreatment with saline ( $\circ$ ,  $n = 11$ ), TNF ( $\bullet$ ,  $n = 7$ ), cyclic mu L tip ( $\blacktriangle$ ,  $n = 5$ ) or cyclic mu scrambl Tip ( $\nabla$ ,  $n = 4$ ) on weight changes (A) and lung mechanics (B-D) in isolated rat lungs before and after (time = 0) alveolar flooding with 2 ml normal saline and subsequent reperfusion for 2 hours. Data points represent mean  $\pm$  SE values; \*  $p < 0.05$  compared to saline group.

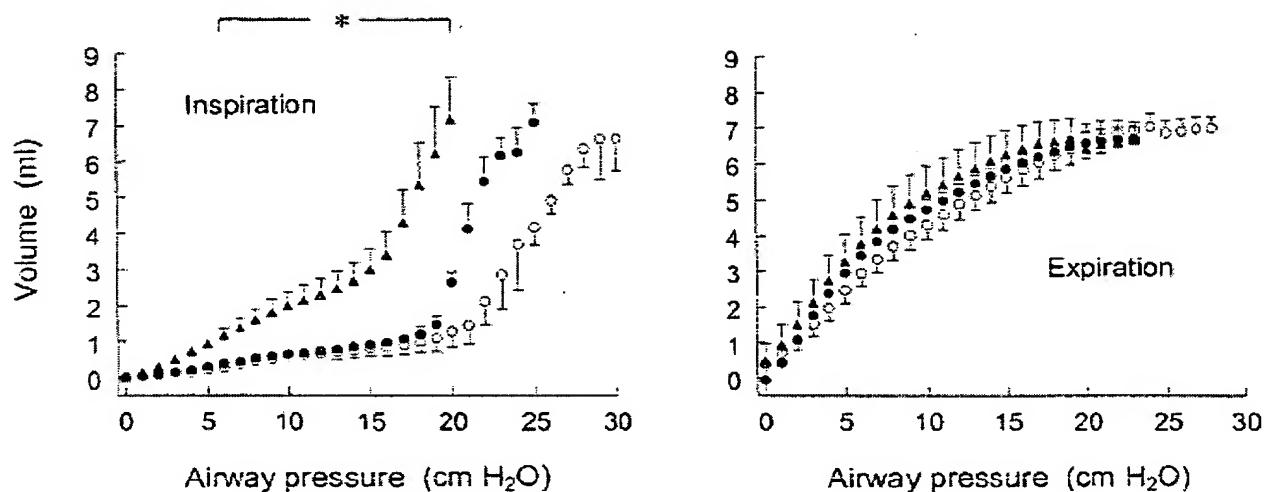


Figure 5:

Effect of intratracheal pretreatment with saline (○, n = 5), TNF (●, n = 4) or cyclic mu Ltip (▲, n = 4) on quasi-static inspiratory (upper panel) and expiratory (lower panel) pressure-volume curves in isolated rat lungs at the end of lung reperfusion for 2 hours following alveolar flooding with 2 ml normal saline. Data points represent mean  $\pm$  SE values; \* p < 0.05 compared to saline.

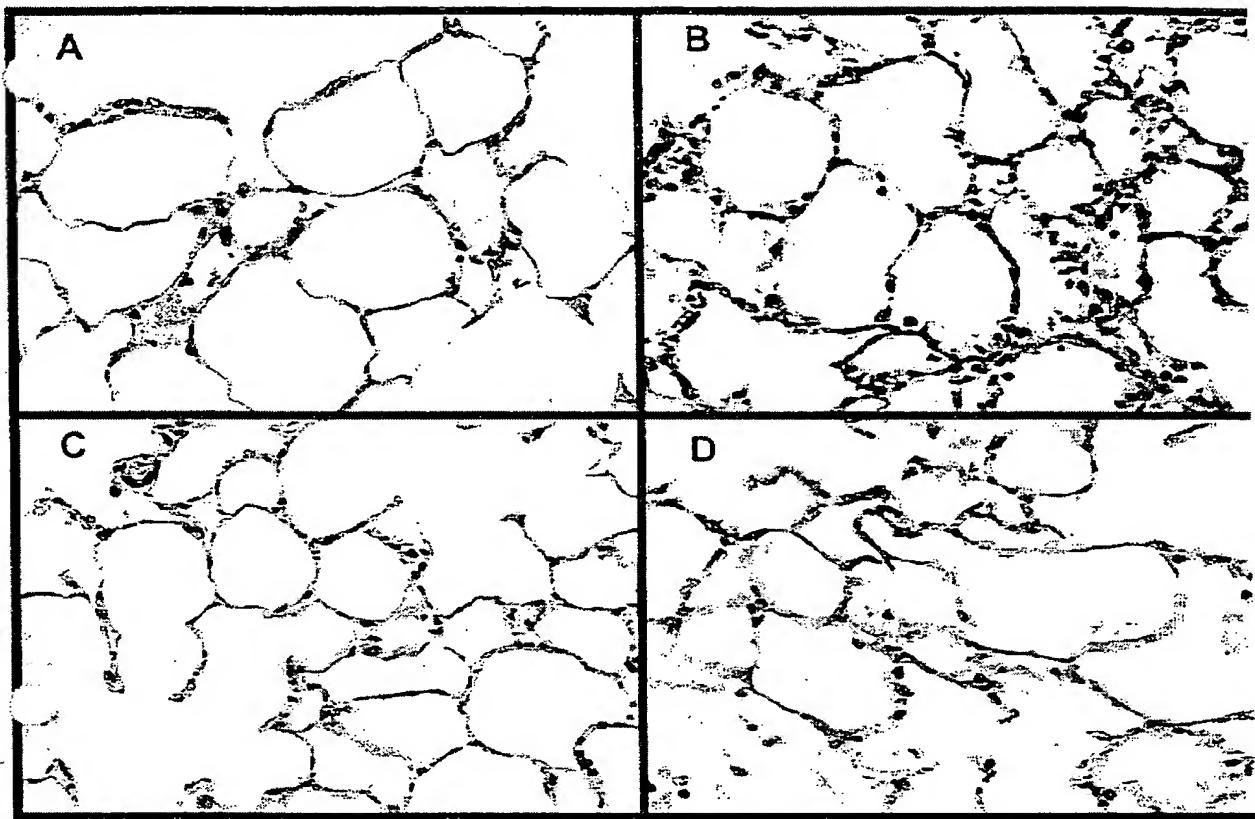


Figure 6:

Lung histology of representative rat lungs pretreated with normal saline (A), TNF (B), cyclic mu Ltip (C), or cyclic mu scrambl Tip (D), followed by alveolar flooding with 2 ml normal saline and subsequent lung reperfusion for 2 hours. Hematoxylin-eosin; magnification: 40x.

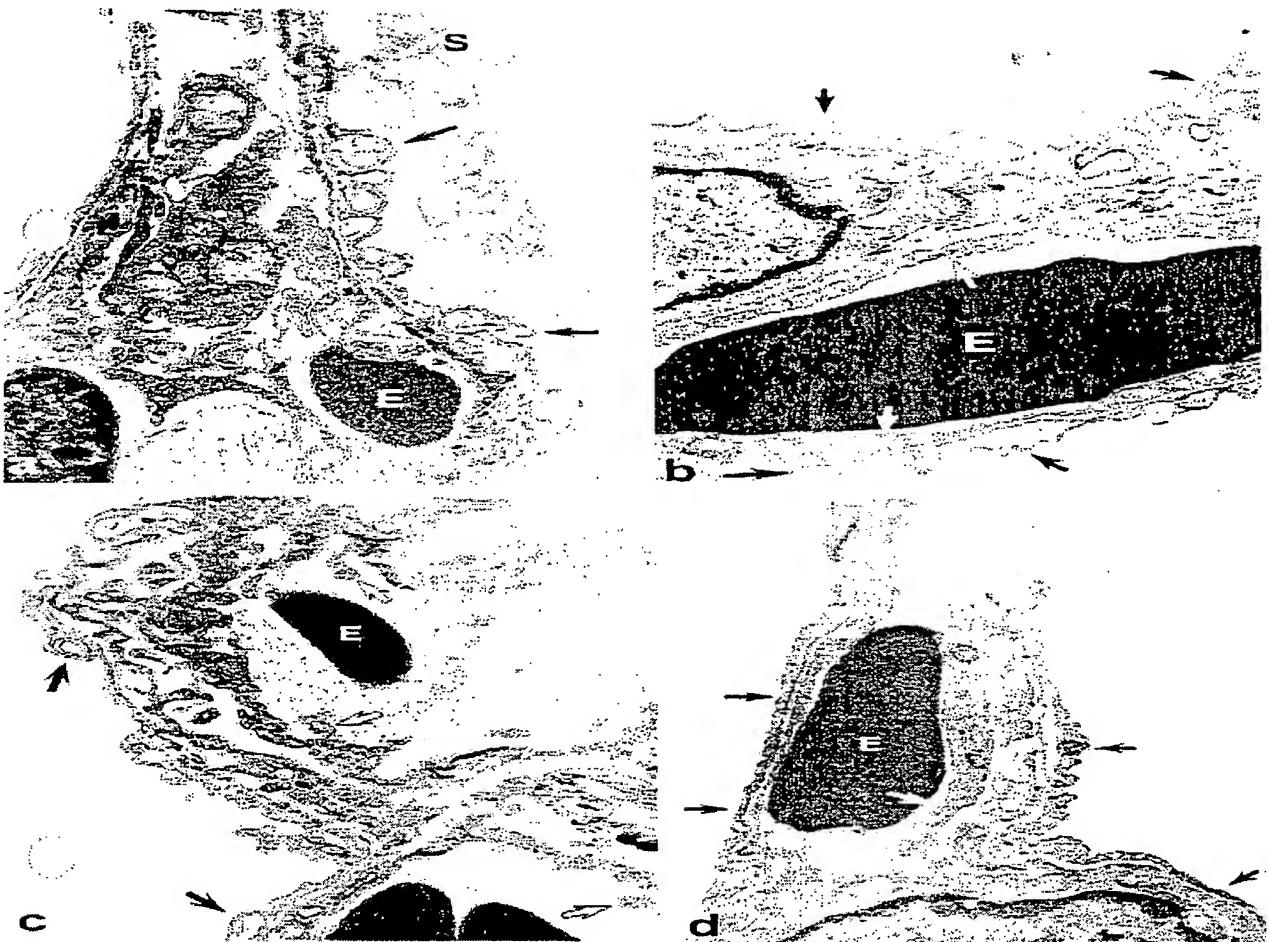


Figure 7:

Electron micrographs illustrating increased alveolar epithelial surface and epithelial blebs in representative rat lungs pretreated with TNF (a), cyclic mu Ltip (b), cyclic mu scrambl Tip (c), or normal saline (d) followed by alveolar flooding with 2 ml normal saline and subsequent lung reperfusion for 2 hours. (a): prominent numerous endothelial flaps (white arrow) protracting in the vascular lumen around an erythrocyte (E). Numerous cytoplasmic blebs of the pneumocyte I (black arrow). Tubular myelin surfactant (S) in the alveolar space. (b): absence of significant endothelial flaps (white arrow) or epithelial blebs (black arrow); slight edema of the pneumocytes type I (black arrow) and endothelial cells (white arrow). (c): endothelial flaps (white arrow) and epithelial blebs (black arrow) less evident than in (a). (d): slight focal edema of the pneumocytes I (black arrow) and endothelial cells (white arrow); minimal amount of flaps or blebs. a-d: original magnification 9800 x.

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Example 5:

**In vitro tissue culture model to examine alveolar epithelial fluid transport**

**Introduction**

TNF- $\alpha$  derived peptides have proven to enhance alveolar liquid clearance in several animal models of non-cardiogenic edema (acute ischemia/reperfusion; lung isotransplantation).

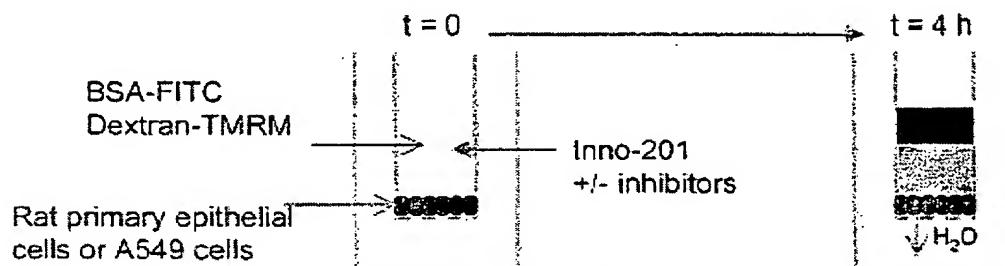
Resolution of alveolar oedema results from the directional water transport across the lung epithelial cell layer. The alveolar epithelium is mainly composed of two major epithelial cell types referred to as the type I and type II cells. Both types express transport proteins and can be involved in the regulation of volume and electrolyte composition of the alveolar lining fluid. The polarized monolayer exhibits a functionally distinct apical and basolateral membrane. There is strong evidence that ion transporters for sodium, chloride as well as the Na/K ATPase pump are important in creating an osmotic driving force across the epithelial monolayer.

**Description of the model**

Airway epithelium is classified as "tight" epithelium, which refers to their degree of paracellular junction tightness, which provides a route for passive ion permeation. In "tight" epithelium the junctions have a low conductance and show high electrical resistance. It is usually assumed that "tight" membranes with regard to ion transport, also have a low water permeability, with *water transport and ion transport being interdependent*.

A tissue culture monolayer system that mimics some aspects of the alveolar epithelial cell layer in lung tissue is created using either freshly isolated rat alveolar epithelial cells or the human epithelial type II-like cell line A549 in a Transwell system.

Water transport from the apical to the basolateral side of the cell layer is monitored by measuring changes in the concentration of fluorescently labeled BSA-Alexa488 and/or dextran-TMRM 70000MW in the upper chamber of the Transwell system. These high molecular weight compounds are not able to passively diffuse (permeate) across "tight" cell monolayers, thus changes in their concentration are due to liquid transport. An increased concentration of the fluorescently labeled molecules in the upper chamber of the Transwell indicates netto water transport from the apical to the basolateral solution.



**Materials and methods**

**TNF- $\alpha$  derived peptides and control compounds**

Table 3 summarizes the characteristics of the TNF- $\alpha$  derived peptides used in this transwell model:

**Table 3:**

IGP859	huTNF	CGQRETPEGAEAKPWYC	Cyclic human Ltip (cycl. hum Ltip)
IGP1811	huTNF	CGQRETPEGAEAKPWY	Linear human Ltip (lin.hum Ltip)
IGP1816	MoTNF	CGPKDTPEGAEELKPWY	Linear murine Ltip (lin. mu Ltip)
IGP1813	huTNF	TPEGAEAC	Linear short tip ( lin. sTip)
IGP 693	huTNF	CTPEGAEAC	Cyclic short tip (cycl. sTip)
IGP 698	moTNF	CGPKDTPEGAEELKPWYC	Cyclic murine tip (cycl. mu Ltip)
IGP1822	huTNF	CGQRETAEGAEAKPWYC	Cyclic human P mutant
IGP1843	huTNF	CGQRETAEGAEAKPWYC	Linear human P mutant

The TNF- $\alpha$  derived peptides were applied to the apical side of the cell monolayer at a concentration of 12.5  $\mu$ M. As a positive control, 100  $\mu$ M terbutaline (sigma) was used.

**Cells**

Isolation of primary lung epithelial type II cells from rat (Dobbs et al., 1986):

ATII cells were isolated from pathogen-free female Sprague-Dawley or Wistar rats weighing 180 to 200 g (obtained from Elevage Janvier). Pooled cells from 3 rats were prepared as follows. Rats were anesthetized intraperitoneally with an injection of pentobarbital (30 mg/kg) and heparin (ip 6125U/g). After a tracheotomy was performed the animal was exsanguinated by cutting the renal artery. Solution II (140 mM NaCl, 5 mM KCl; 2.5 mM sodium phosphate buffer, 10 mM Hepes, 2mM CaCl<sub>2</sub>, and 1.3 mM MgSO<sub>4</sub>, pH 7.4) was perfused through the ventilated lungs via the pulmonary artery to clear the vascular space of blood. The lungs were removed from the thorax, filled with DMEM and lavaged to total lung capacity 7 times with solution I (140 mM NaCl, 5 mM KCl; 2.5 mM sodium phosphate buffer, 10 mM Hepes, 6mM D-glucose and 2 mM ethylene glycol-bis (Beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid) and 3 times with solution II. Lungs were then filled with 5 ml elastase solution (Worthington Biochemical Corporation; 1.7 mg/ml in solution II) and incubated in a water bad at 37°C for 10 min, followed by 2 additions and 10 minute incubations of 5 ml elastase solution. The lungs were minced in the presence of DNase I, and 5 ml of FBS were added to stop the effect of elastase. The lungs were then sequentially filtered through 150- and 30  $\mu$ m nylon mesh. The filtrate was centrifuged at 130 g for 8 min. The cell pellet is resuspended in DMEM and the cells are seeded in bacterial plates and incubated at 37°C to remove lung tissue macrophages by differential adherence. The supernatant is collected, the cells are washed and plated at 1.2 x 10<sup>6</sup> cells/cm<sup>2</sup> in DMEM high glucose with 1%BSA. Penicillin/streptomycin, 5  $\mu$ g/ml gentamycin. Every 2 days the medium is changed. The yield of rat alveolar epithelial type II cells is between 2 and 4 X 10<sup>7</sup> cells/rat. (Protocol approved by the local animal ethical committee (02.R005.1))

Human A549 cells ATCC CCL-185

A549 human type II-like epithelial lung cells were purchased from the ATCC. The cells were grown as monolayers in tissue culture flasks in Ham's F12K medium supplemented with 10% FBS.

Transwell culture and experiments

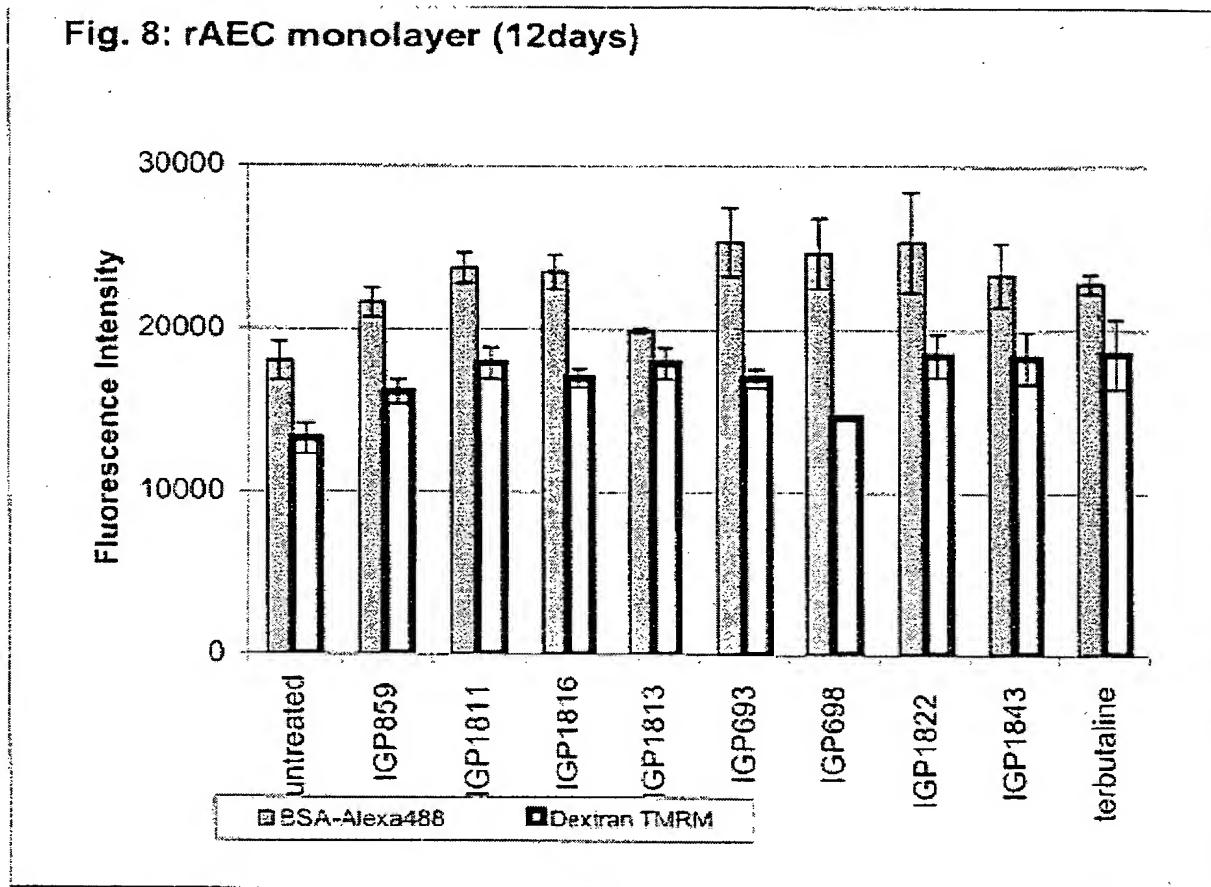
Freshly isolated rat lung epithelial type II cells were seeded on the upper surface on permeable filters in Transwell tissue culture plates. Upper and lower chamber of the plates are separated by a translucent polyethylene terephthalate (PET) (Falcon) or polyester (Costar) filter with a 0,4  $\mu\text{m}$  pore size.  $1,2 \times 10^6$  cells/cm $^2$  were seeded on the filter in DMEM with 1%BSA and cultured for 8-12 days to obtain confluency and differentiation to a polarized monolayer.

A549 cell monolayers in tissue culture flasks were harvested with trypsin (0.25%) and EDTA (0.1%) in PBS, centrifuged and resuspended in fresh medium prior to culture on the upper surface on permeable filters in Transwell tissue culture plates.  $10^5$  cells/cm $^2$  are seeded in Ham's F12K medium with 10% FBS and cultured for 6-10 days to obtain confluency and differentiation to a polarized monolayer.

Permeability of the cell monolayer was checked in 3 control transwells by dextran blue 2000. Dextran Blue 2000 (1%) was added to the upper chamber of the transwell culture and incubated at 37°C for 2 hours. After incubation the amount of dextran blue in the upper and lower chamber of the transwell is determined, measuring the absorption at 620 nm Only when there was no permeation of dextran blue across the cell monolayer, the transwells were used for analysis of water transport across the cell monolayer.

12,5  $\mu\text{M}$  of INNO201 and its derived peptides or 100  $\mu\text{M}$  terbutaline were added to the apical side of the monolayer, in growth medium with 1%BSA. 50  $\mu\text{g}/\text{ml}$  BSA-Alexa488 and 20  $\mu\text{g}/\text{ml}$  Dextran TMRM. In the lower chamber growth medium with 1%BSA was used. All conditions were performed in triplicate. The transwells were incubated for 4 hs at 37°C. Fluorescence intensity of the upper chamber solutions was measured on a plate reader (Packard).

Fig. 8: rAEC monolayer (12days)



## Results

Fig 8 summarizes the results of a transwell experiment with a cell monolayer from primary rat alveolar epithelial cells (rAEC). Freshly isolated rAEC were grown in the transwell culture for 12 days. The results clearly demonstrate that all the peptides tested, including the short peptides (8AA -IGP693; 7AA - IGP 1813) and the P-mutants (IGP 1822 and IGP 1843) were equally effective in inducing netto water transport from the apical to the basolateral side of the epithelial monolayer.

8. I believe that Example 1 above clearly demonstrated that hum Ltip, as a cyclic peptide or in its linear conformation, as well as the shorter peptide Stip are capable of clearing alveolar fluid content ex vivo after intratracheal instillation of the molecule when testing in an artificial model of hydrostatic edema simulating the symptoms seen in heart failure. The lectin-deficient peptide (hum mut Tip), wherein the TPEGEA is replaced by APAGAA is inactive in clearing alveolar fluid content, again demonstrating that the residues T,E,E are essential for the effect.

9. In a warm ischemia/reperfusion model (Example 2), animals treated with the hum Ltip peptide have significantly much better pO<sub>2</sub> levels compared to the control animals or animals treated with the hum mut Tip peptide. This clearly indicates that the hum Ltip treated animals are suffering less from fluid overload and edema has been treated.

10. Hum Ltip given by intratracheal application efficiently restores the lung function of the donor lung in a transplantation model (Example 3).

11. The results obtained in the in situ mouse lung model (Example 4) again clearly indicate that TNF- $\alpha$  mediates fluid resorption by means of a TNF-receptor

independent mechanism. It is further also demonstrated that cyclic murine Ltip induces a significant weight loss in flooded perfused rat lungs as well as an improvement of respiratory parameters, without inducing a pro-inflammatory reaction in the lung.

12. In Example 5, several TNF-derived peptides (human Ltip, murine Ltip, the short peptide Stip and a mutant hum Ltip peptide in which the P residues at position 106 is replaced by an A residue (P106A), both in their cyclic and linear conformation) were tested in an invitro tissue culture model to examine their activity on alveolar epithelial fluid transport. All tested peptides were equally effective in inducing netto water transport from the apical to the basolateral side of the epithelial monolayer.

13. It is my view that one of ordinary skill could make and use the claimed invention.

I declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By:   
Lucie Fransen

Date: 21 july 2004

## CURRICULUM VITAE

### Identification :

Name : FRANSEN Lucia, Maria, Leona.

Date of birth : June 12, 1956.

Sex : Female

Civil status : unmarried

Nationality : B.

Home address : Kasteeldreef 45  
B-8020 Hertsberge  
Belgium  
Tel: 050 27 00 24  
Mobile: 0495 69 51 09  
e-mail: [lucie.fransen@skynet.be](mailto:lucie.fransen@skynet.be)

Business address: Innogenetics  
Industriepark 7  
B-9052 Zwijnaarde  
Belgium  
Tel: 09 324 20 06  
e-mail: [luciefra@innogenetics.com](mailto:luciefra@innogenetics.com)

### Education and qualifications :

1962-1974      **Primary Education and High School**,  
Latin and Sciences at St. Pieters Institute in Ghent.

1974-1978      **Biological Sciences**  
State University of Ghent

1978            **University Licence of Biological Sciences**,  
with high distinction, State University of Ghent.

Thesis in the Moleculat Biology (Prof. W. Fiers)  
: "Heterokaryons of eukaryotic cells: fusion techniques and quantitative analysis"

1978 - 1982     **Graduate student** in the Laboratory of Molecular Biology of Prof. W. Fiers.  
State University of Ghent.

1983            **Ph.D. - Biology**: Highest distinction.  
State University of Ghent - Laboratory of Molecular Biology.  
Director and promoter: Prof. W. Fiers  
Specialisation: Molecular Biology.

Thesis: "Protein phosphorylation as a possible primary factor in the SV-40 induced transformation process".

### Scientific memberships

Member of the Belgium Immunological Society  
Member of the International Cytokine Society  
Member of the International Interferon Society

### Languages

Dutch: mother language  
English: fluent writing of scientific articles and fluent presentations at symposia  
French: speaking and writing

#### **Professional experience**

1982 -1985 : Postdoctoral fellow in the laboratory of Biogen, research laboratory of Biogen S.A.  
Plateaustraat 22, B-9000 Ghent, Belgium.  
Title: Projectleader of the TNF molecular clonings program  
Supervising 5 people and reporting directly to the Biogen Headquarters in Geneva

1985 – 2004

1985	Member of the team initiating INNOGENETICS n.v. Industriepark Zwijnaarde 7, Bus 4 - B-9052 Zwijnaarde (Ghent), Belgium. Responsible for the scientific and technical build up of the Research Laboratory of the company .
1985 – 1986	Project leader of the project: "Molecular cloning of the bovine activin A and B chains" and responsible for fund raising.
1986	Research contract with Japanese Company (Kaneka)
1986 - 1990	Project leader of the Kaneka_Innogenetics contract. Supervising and co-ordinating 15 research collaborators and responsible for the direct reporting to Kaneka
1990	Re-organisation of the Research Department of the company into Departments and Projects
1990 – 1994	Interim Department Manager Molecular Biology. Responsible for the scientific and technical building up of the Department. Supervising and co-ordinating the work of 7 department members.
1990 – 2000	Project leader of an Innogenetics' interdepartmental project. "Molecular cloning and biological activity of a new macrophage derived protein." Supervising and co-ordinating the work of 12 project team members and the collaboration with the VUB . Responsible for fundraising (IWT) and yearly reporting. Development of the technical know-how of the Department of Cellular Immunology
1992 – 1993	Project leader of a project in collaboration the KULAK and UIA "Isolation, characterisation and cloning of a neutrophil degranulation inhibitor"
1992 – 2000	Research project leader of the TNF-lectin carbohydrate project in collaboration with the VUB and the University of Geneva
1999 – 2001	Project leader of a IWT sponsored project in collaboration with two different research groups of the University of Ghent "Differential expression of human interleukin 5 receptor a-subunit isoforms in nasal polyposis"
2000 – 2003	Therapeutic Project Manager Managing an international pre-clinical development team. <ul style="list-style-type: none"><li>- Responsible for the co-ordination of the global development of the compound in relation to product development and pre-clinical pharmacology</li><li>- Organizing an international Advisory Board</li><li>- Interaction with International Marketing to analyse opportunities in the field of edema on the basis of Disease magnitude, Current treatment approaches, Market analysis,</li></ul>

## Competitive landscape, Unmet needs and Market drivers

2003 – until now      Therapeutic Program Manager  
                            Managing and coaching Therapeutic Project Managers

- Responsible for the co-ordination, budgeting and strategic planning of different Therapeutic Projects
- Member of the Therapeutic portfolio decision committee
- Therapeutic evaluation and assessment of Innogenetics' therapeutic projects
- Initiators and moderator of a Therapeutic Advisory board
- Involved in the in-licensing project of Therapeutic vaccination portfolio

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"Metabolic turnover of phosphorylation sites in simian virus 40 large T antigen"  
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**Patents:**

Co-inventor on the following patent applications

1. "Purification, production and use of Tumor necrosis factors"

EUROPEAN PATENT APPLICATION No 86900138.8  
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2. "Combination of tumor necrosis factors and antibiotics and methods for treating tumors"

EUROPEAN PATENT APPLICATION No 87900104.8  
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3. "Purification, production and use of tumor necrosis factors"

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5. "New polypeptides, a process for preparing them and their use as inhibitors of degranulation"

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